ELECTRON MICROSCOPY OF OSTEOCLASTS IN HEALING FRACTURES OF RAT BONE

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ABSTRACT

Osmium-fixed, undecalcified, callus tissue from healing fractures of rat tibias was sectioned with a diamond knife for study with the electron microscope. Large multinucleated cells were found adjacent to bone. A characteristic labyrinthine infolded border was consistently seen in parts of the cells close to the bone surface. The innermost parts of this "ruffled border" gave rise to vacuoles. The bone surface was always disrupted under the "ruffled border" of the cells. Needle-like crystals were seen at the osseous fringe, within folds in the ruffled border as well as within vacuoles deeper in the cells. Collagen fibers denuded of crystals were never observed. Mitochondria, containing clusters of fine granules, were abundant. The part of the cell away from bone contained rough endoplasmic reticulum and the cell membrane was thrown into irregular microvilli. These observations are discussed in relation to current concepts of osteoclastic resorption of bone.

Giant cells adjacent to bone had been described by Robin as early as 1864 (19), but it was Kölliker (12) who gave them the name "*Ostoklast*" and proposed that they are the agents of bone resorption. Much debate has followed concerning the function of these cells.

Osteoclasts are giant cells varying from about 20 to 100 μ in their long axis (9). The larger cells contain as many as 100 nuclei. Their cytoplasm is claimed to be moderately basophilic and to become moderately acidophilic with age (9). They are located in areas of bone resorption, usually in cavities or pits (Howship's lacunae) on the surface.

Since the recent reviews of Hancox (8 and 9) and McLean (14), further important contributions have been made. At a recent symposium on parathyroids (6) Goldhaber and Gaillard independently showed time-lapse cinemicrographic studies of osteoclasts in tissue culture. They gave convincing evidence that osteoclasts participate actively in bone resorption.

Electron microscopic studies by Scott and Pease (21) revealed osteoclasts in the epiphyses of kitten humeri. They described these cells has having a characteristic "ruffled border" containing crystals between folds of the cell membrane. They suggested that a "collagen dissolving substance," secreted through the ruffled border, releases crystals originally present in the organic matrix. The crystals are then said to be phagocytized by the osteoclasts and later digested. Cameron and Robinson (2) also used the electron microscope and found a somewhat similar picture of osteoclasts in the epiphyses of newborn infants. Their report fails to mention a "ruffled border," but stresses a vacuolated appearance of the cytoplasm.

The present work stems from an electron microscopic study of osteo-lathyrism in rats (5). Our observations on osteoclasts in healing fractures of

rat tibias support some of the views of Scott and Pease (21). These and other observations on the submicroscopic morphology of osteoclasts not mentioned by them are presented below.

MATERIALS AND METHODS

Male Sprague-Dawley rats[1] weighing 100 to 107 grams had their left tibias fractured according to the technique of Selye (20). After the operation each rat received 150,000 units of procaine penicillin subcutaneously. Sixteen days later the rats were killed by decapitation. The fracture calluses were bisected with a razor blade, and one-half of each was fixed in formol-alcohol for light microscopy. Portions of the other halves were taken for electron microscopy. The specimens for light microscopy were decalcified in 5 per cent formic acid after fixation, and then double-embedded in celloidin-paraffin. Sections were cut at six microns and some were stained according to the Gomori aldehyde-fuchsin technique, counterstained with orange-G and hematoxylin, others were stained with hematoxylin and eosin.

The pieces taken for electron microscopy were fixed for 2 hours in cold 1 per cent buffered (pH 7.3) OsO_4 fixative containing sucrose (3), subsequently dehydrated in a series of graded ethanol solutions and embedded in a mixture of methyl- and butyl-methacrylate (1.9). Thin sections were cut with a Sorvall-Porter-Blum microtome fitted with a diamond knife (4). Sections were sandwiched between formvar films on copper grids and observed with an RCA EMU-3C electron microscope at 50 KV with 50 μ platinum objective aperture. Micrographs were taken at original magnifications of 2000 to 37,000, and subsequently enlarged photographically.

[1] Obtained from the Charles River Laboratories, Boston.

OBSERVATIONS

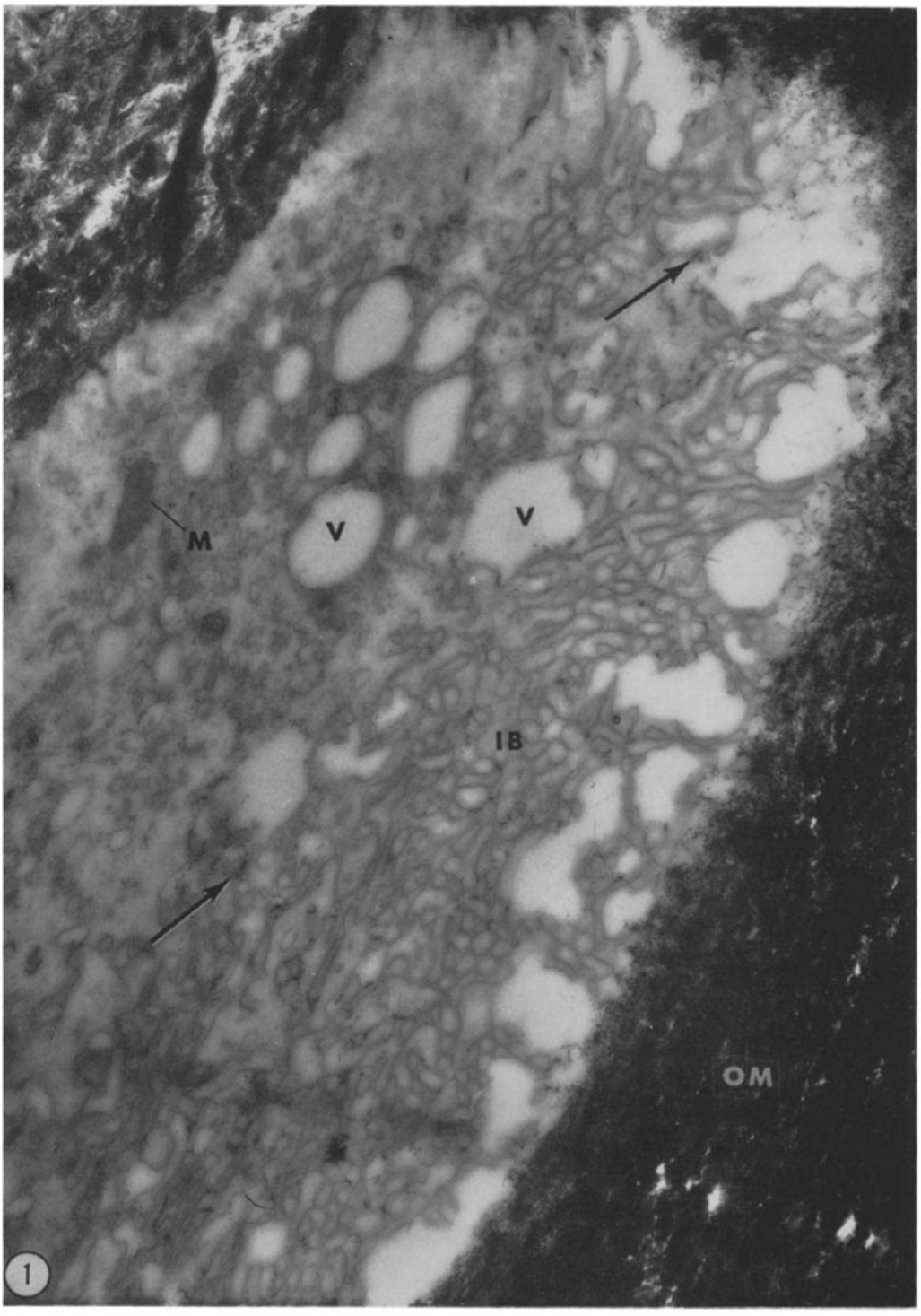
Light Microscopy: Light microscopic observations of the fracture area revealed the classical picture of bone healing. The reader is referred to the work of Ham and Harris (7) for a detailed description of this process. For the present purposes it will suffice to note that there is deposition and remodeling of bone in the healing process. The remodeling involves resorption. Hence, it is not surprising to find osteoclasts in the callus.

The cytoplasm of osteoclasts varied from basophilic to moderately acidophilic. The nuclei were fairly large, sometimes almost vesicular and varied in number. Most of the cells examined had a group of vacuoles in their cytoplasm, usually on the side next to the bone. Occasional osteoclasts were found to contain an ingested cell within a vacuole. The so called "striated border" was rarely seen.

Proximal Cell Boundary: Low-power micrographs showed large cells adjacent to bone (Fig. 1). These cells (Figs. 1 to 3, 7, 8, and 12) presented a more or less well developed "ruffled border" as described by Scott and Pease. Only those parts of the cell immediately adjacent to bone showed the highly complicated infolding of the plasma membrane. Usually, the border was ruffled only along part of the junction between cell and bone. Thus, in Fig. 1, the part of the cell along the cell-bone junction at the upper left hand corner of the micrograph does not show a ruffled border. Corresponding with the change from a ruffled to a relatively smooth border was a change in the nature of the osseous fringe. The morphology of the ruffled border was quite variable, ranging

FIGURE 1

Low power electron micrograph of undecalcified section of callus showing a portion of an osteoclast adjacent to bone. The calcified bone (*OM*) is the electron dense area seen in the upper left, upper right and lower right corners of the picture. As one follows the edge of the bone from the left, around to the top and down to the bottom of the picture, one sees a change in the appearance of the fringe. Beginning at the top, the part of the osseous fringe on the right is not as sharply defined as that on the upper left. Corresponding with this change in character of the bone surface, is a change in the cell boundary. Note the extremely tortuous infolded border (*IB*). This is the "ruffled border" of Scott and Pease. Fine, needle-like crystals (at arrows) can be seen along the edge of the bone, within the folds of the cell membrane and also within vacuoles (*V*) deeper in the cell. A mitochondrion is pointed out at *M*. Note absence of naked collagen along edge of bone. Magnification, 13,200.


M
V
V
IB
OM
1

from a very tight infolding (Fig. 14) to a very loose, open infolding (Fig. 15). The surface of bone under the smooth boundaries of the cells was itself relatively smooth and intact (Figs. 1 and 4), whereas that under the infolded border was disrupted and showed many loose crystals (Figs. 1 to 4, 6). Varying amounts of loose crystals were also found between membranes in the labyrinth formed by the complicated infolding of the plasmalemma (Figs. 1 to 3, 7, 12) as well as in vacuoles deeper in the cell (Fig. 1). Some of these vacuoles were observed to communicate with the ruffled border, thus forming internal dilatations of the narrow intermembranous spaces.

In certain areas, the plasma membrane was covered by an outer, moderately electron-dense, diffuse layer (Figs. 2, 9). Most regions of resorption showed no signs of organic matrix (Figs. 1 to 3). Others had very fine filaments randomly scattered in spaces between the bone and the cell (Figs. 6, 8). Whether these filaments were present in the living tissue or represent once dispersed material precipitated during preparation is not known. In either case, these filaments could represent the remains of destroyed collagen.

Distal Cell Boundary: The distal profile of the cell membrane, that is to say, that part away from the bone matrix, was observed in only two osteoclasts. In both cases, the border showed irregular microvilli (Fig. 5). Judging from these very limited observations, the distal parts of the cell are richer in endoplasmic reticulum than the proximal parts.

Central Area: Double-membrane–bounded nuclear profiles varied from regular smooth ovals to very irregular shapes (Fig. 10). Plasma membranes were never found separating these nuclei. As many as 7 nuclear profiles were found in a single section of an osteoclast. The usual number was 3 or 4.

Golgi vesicles and membranes were found near the cytoplasm. Many contained granules (Figs. 10 to 12). At higher magnification these granules were seen to be clusters of finer particles (Fig. 13). Some mitochondria presented circular profiles of internal membranes, thus giving evidence of the presence of villiform as well as lamelliform cristae.

DISCUSSION

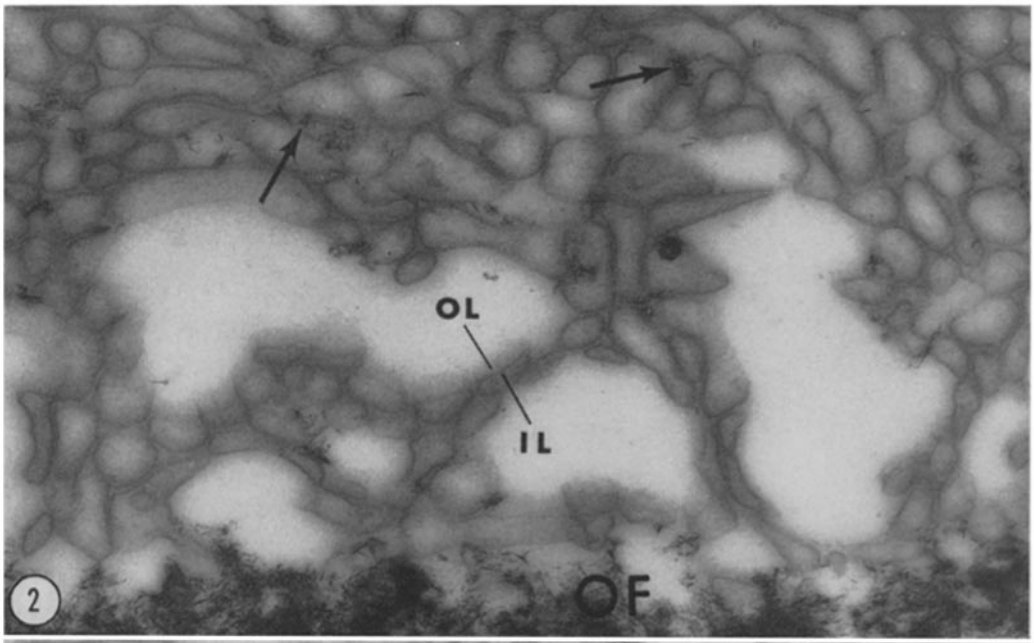
In contrast to the view of McLean and Urist (17, p. 66), it seems evident from the work of Scott and Pease (21), that of Cameron and Robinson (2), and the present study, that osteoclasts phagocytize crystals of bone salts and that these crystals are very probably free of collagen. Observations with the light microscope never reveal the presence of these crystals in osteoclasts; however, bone salts have been described in neighboring macrophages (16). Although bone salts have never been demonstrated in osteoclasts by light microscopy, organic components of bone matrix have been reported in vacuoles within osteoclasts (10, 11, 13). This discrepancy between studies with the light microscope and those with the electron microscope can be attributed to the greater resolving power of the latter. The amount of solid material phagocytized by osteoclasts is not great. In some instances, the process could probably best be described as pinocytosis since the greater part of vacuoles are optically empty, debris taking up only a small proportion of the total volume.

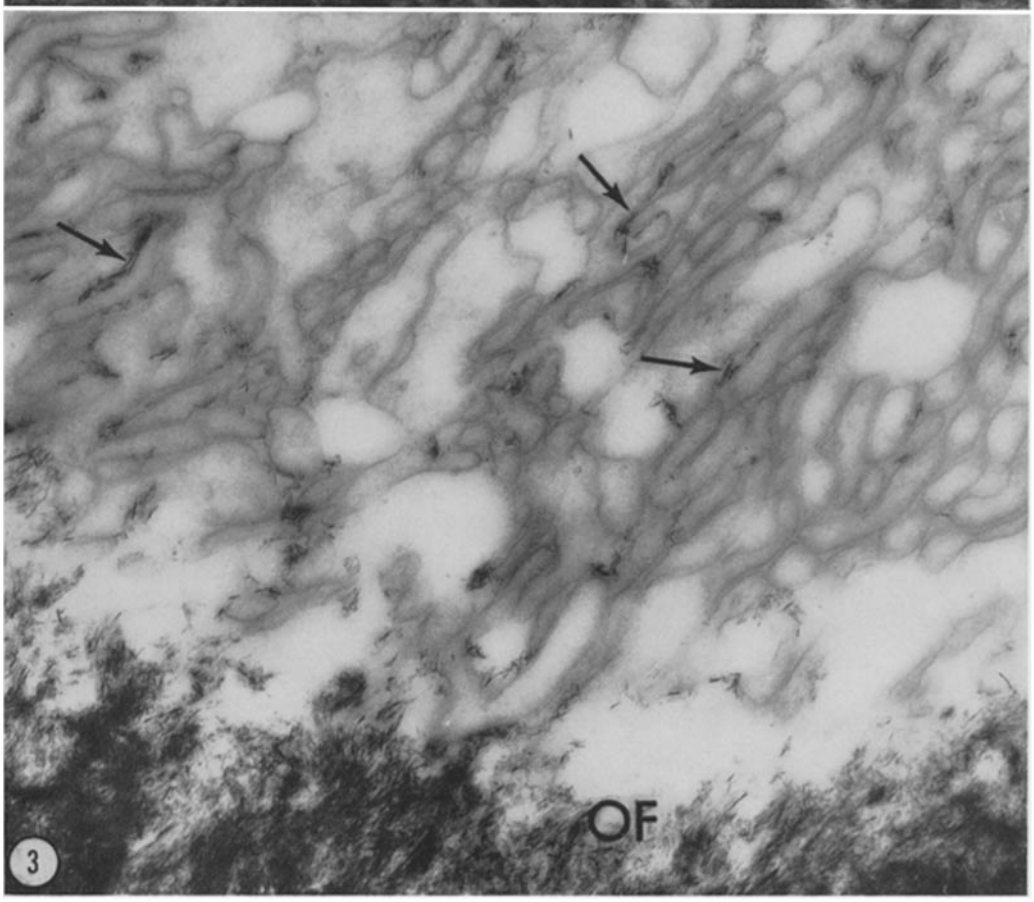
FIGURE 2

Detail of the ruffled border showing osseous fringe (*OF*), crystals at arrows, inner, sharply defined plasma membrane (*IL*), and outer diffuse layer covering it (*OL*). Cell processes are seen to be in intimate contact with the material of the osseous fringe. Note absence of collagen. Magnification, 34,000.

FIGURE 3

Detail of ruffled border. This was printed to emphasize the presence of crystals within the folds of the ruffled border. Some of the crystals are pointed out by arrows. Similar crystals are seen along the osseous fringe (*OF*). Note absence of collagen. Magnification, 34,000.


OL
IL
OF
2


OF
3

Mature collagen fibers free of crystals at the surface of bone in contact with the active portions of osteoclasts were never seen. This fact argues against the view that dissolution of crystals precedes the destruction of the organic matrix in osteoclastic resorption. Mature collagen fibers were observed in other parts of the same sections but not in association with osteoclasts. Their absence under the osteoclasts was, therefore, real and not a false impression gained as a result of failure to stain with osmium. If this fact were considered alone, it would seem that, in osteoclastic resorption, organic and inorganic phases of bone matrix are removed simultaneously as first hypothesized by Kölliker (12), emphasized by McLean and Bloom (15, p. 350) and others, and presently generally accepted.

However, free crystals were consistently present at the osseous fringe, as well as within folds of the ruffled border and in vacuoles deeper in the cell. This can only mean that the organic matrix is disrupted first, and that this frees crystals which are then phagocytized by the osteoclast. Such a sequence is in agreement with that proposed by Scott and Pease (21).

The present observations do not give evidence of a mechanical disruption of bone by osteoclasts, but rather they are consistent with the hypothesis of a chemical agent or agents of bone resorption produced at least in part by the osteoclast itself. The osteoclast probably secretes a substance (or substances) which lyses collagen and depolymerizes the amorphous ground substance and another which chelates calcium (14). Differences in rates of reaction of these hypothetical substances could account for the fact that collagen destruction always appears somewhat more advanced than does solubilization of the bone salts. An osteoclast or a part of an osteoclast may then contain high or low concentrations of free crystals, depending on the activity of the ruffled border. A rapid phase of activity would allow the cell to take in crystals before they had time to dissolve. Slow activity would result in pinocytosis of already dissolved bone salts.

It must be emphasized that the crystals within osteoclasts are thought to be bone salts on the basis of morphology and not on the basis of electron diffraction. The fact that they appear only in proximity to diffuse, disrupted bone surfaces and within and close to the ruffled border also supports this identification.

In both instances in which the distal parts of the osteoclast were observed, a zone rich in rough endoplasmic reticulum was seen (Fig. 5). Rough endoplasmic reticulum was rarely seen in other parts of these cells, especially in those parts close to the bone surface. Admittedly, these observations are very limited, but they are reinforced by the finding that most osteoclasts have RNA concentrated in a zone of cytoplasm away from the bone (18).

The large numbers of mitochondria observed in osteoclasts is consistent with the finding that these cells are high in cytochrome oxidase and succinic dehydrogenase, a finding which suggests a high metabolic activity (1).

Although granules of various shapes and sizes have been observed in a variety of other cell types, the presence of intramitochondrial clusters in osteoclasts is a feature that has not been reported before. Definitive interpretation of these clusters must await further work.

Hancox (9) suggests that vacuoles seen with the light microscope are secretory in nature. The presence of crystals within as well as communicating channels between vacuoles and the ruffled border indicates that at least some are phagocytic

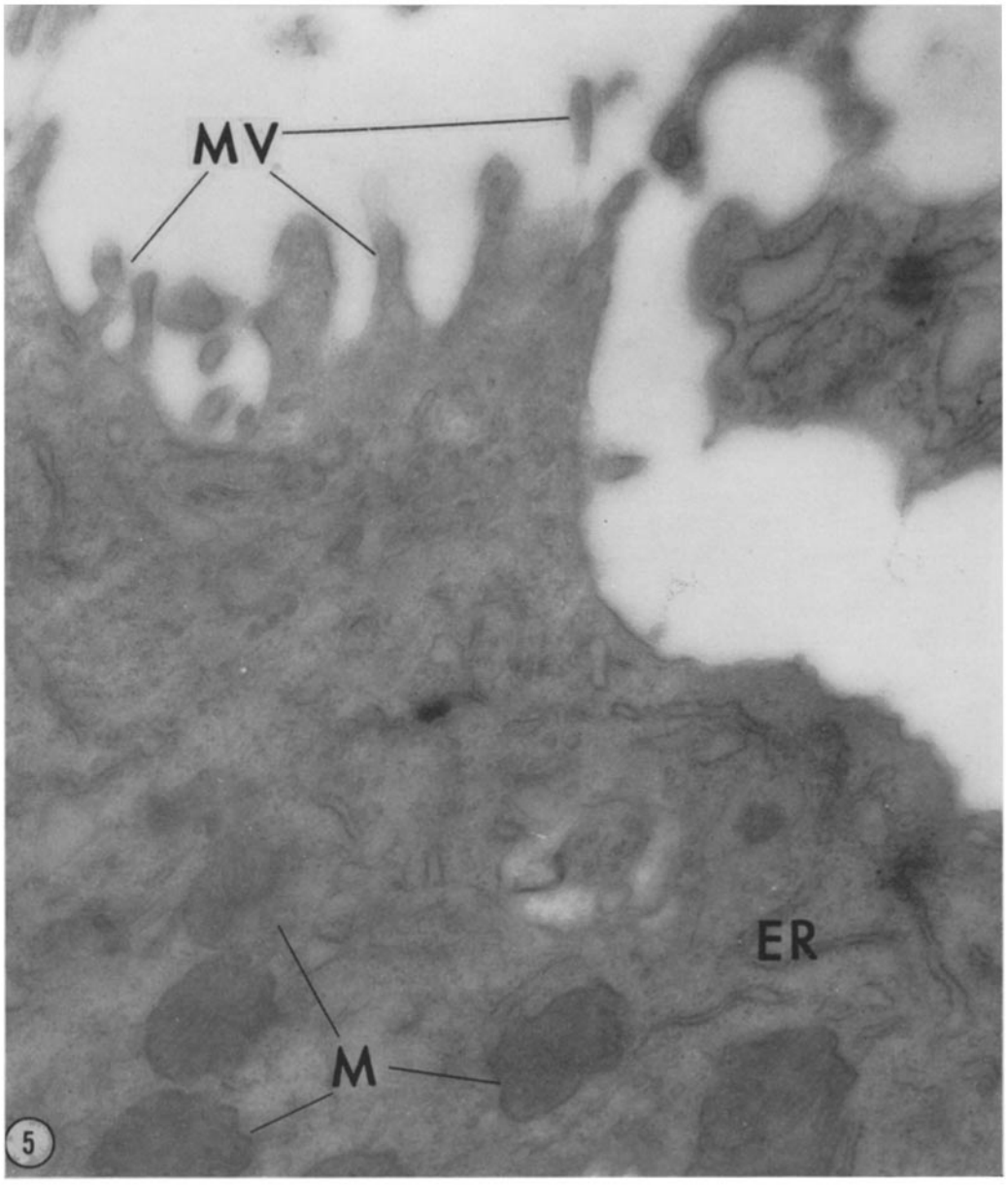
FIGURE 4

Detail of junction between intact osseous fringe (*IOF*) and disrupted osseous fringe (*DOF*). Arrows indicate portion of plasma-membrane. Magnification, 34,000.

FIGURE 5

Border of osteoclast away from the bone surface. Irregular microvilli can be seen at *MV*, mitochondria at *M*, and rough endoplasmic reticulum at *ER*. Magnification, 34,000.


DOF
IOF
4


MV
ER
M
5

rather than secretory. Whether vacuoles remain in communication with extracellular space via the ruffled border, or are eventually pinched off is impossible to determine without observing serial sections. This was not attempted in the present study.

The present study confirms the work of Scott and Pease (21) and extends it to osteoclasts in the callus of healing fractures. Furthermore, features not mentioned by Scott and Pease, such as the intramitochondral granule clusters, villiform cristae, variations in morphology of the "ruffled border," endoplasmic reticulum concentrations at the pole of the cell away from the bone surface as well as the presence of microvilli on this surface, have been demonstrated by the present work.

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FIGURE 6

High magnification micrograph of osseous fringe (*OF*) under active portion of osteoclast. Arrows point to some of the characteristic needle-like crystals found in bone. The faint line at (*CR*?) may represent a remnant of the fibrous component of the organic matrix. Magnification, 90,000.

FIGURE 7

High magnification micrograph of a field within the ruffled border of an osteoclast. Large arrows point out crystals identical in size and shape to those in and around osseous fringe. Small arrows point out plasma membrane. The shafts of the small arrows are within cytoplasm. Space between small arrow heads is extracytoplasmic. Magnification, 90,000.


OF
CR?
6

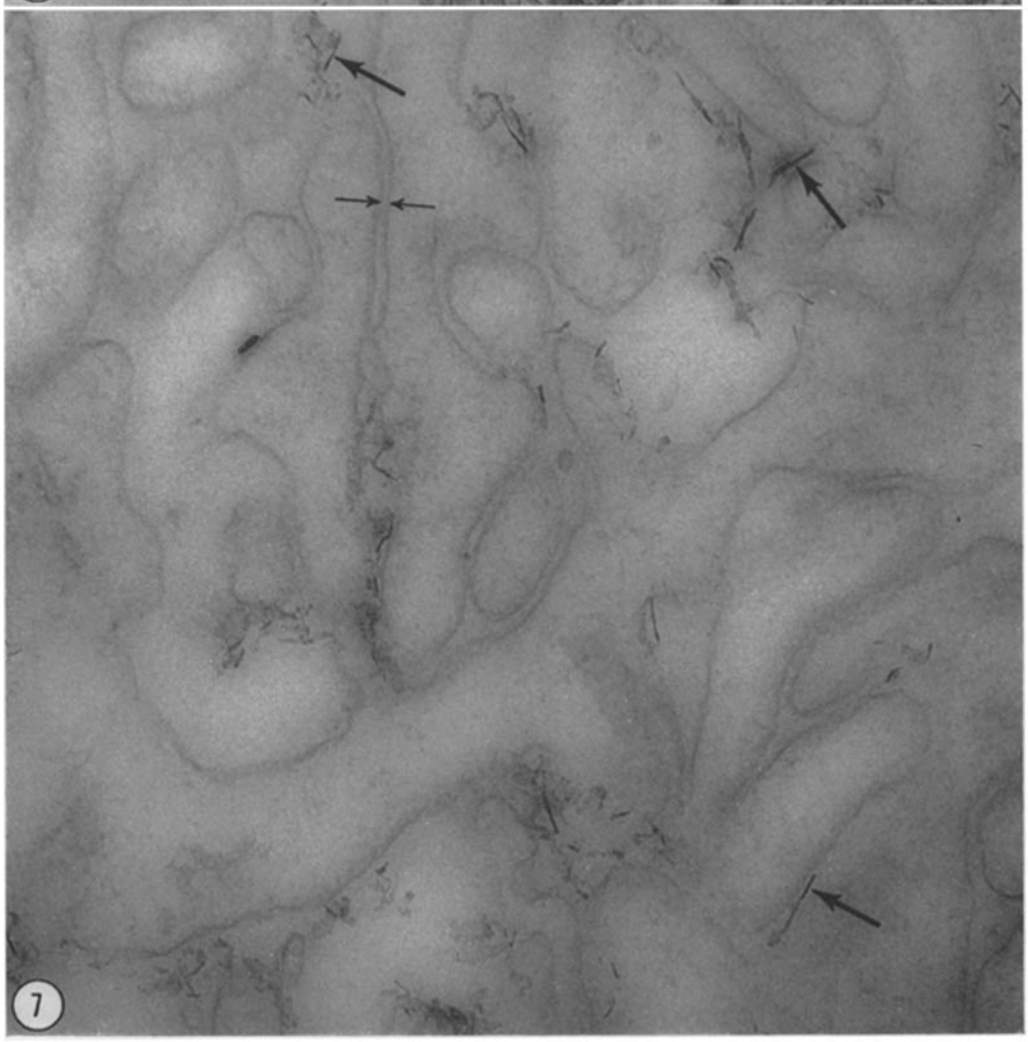
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FIGURE 8

Spider-like processes arising from cell body above and contacting osseous fringe (*OF*) below. Arrows point out fine filaments between processes and within vacuole-like spaces. The edge of a nucleus (*N*) can be seen at the extreme upper left of the picture. Magnification, 34,000.

FIGURE 9

Higher magnification of area at *P*, Fig. 8. *IL*—plasma membrane; *OL*—moderately electron opaque, diffuse material covering plasmalemma. Magnification, 68,000.

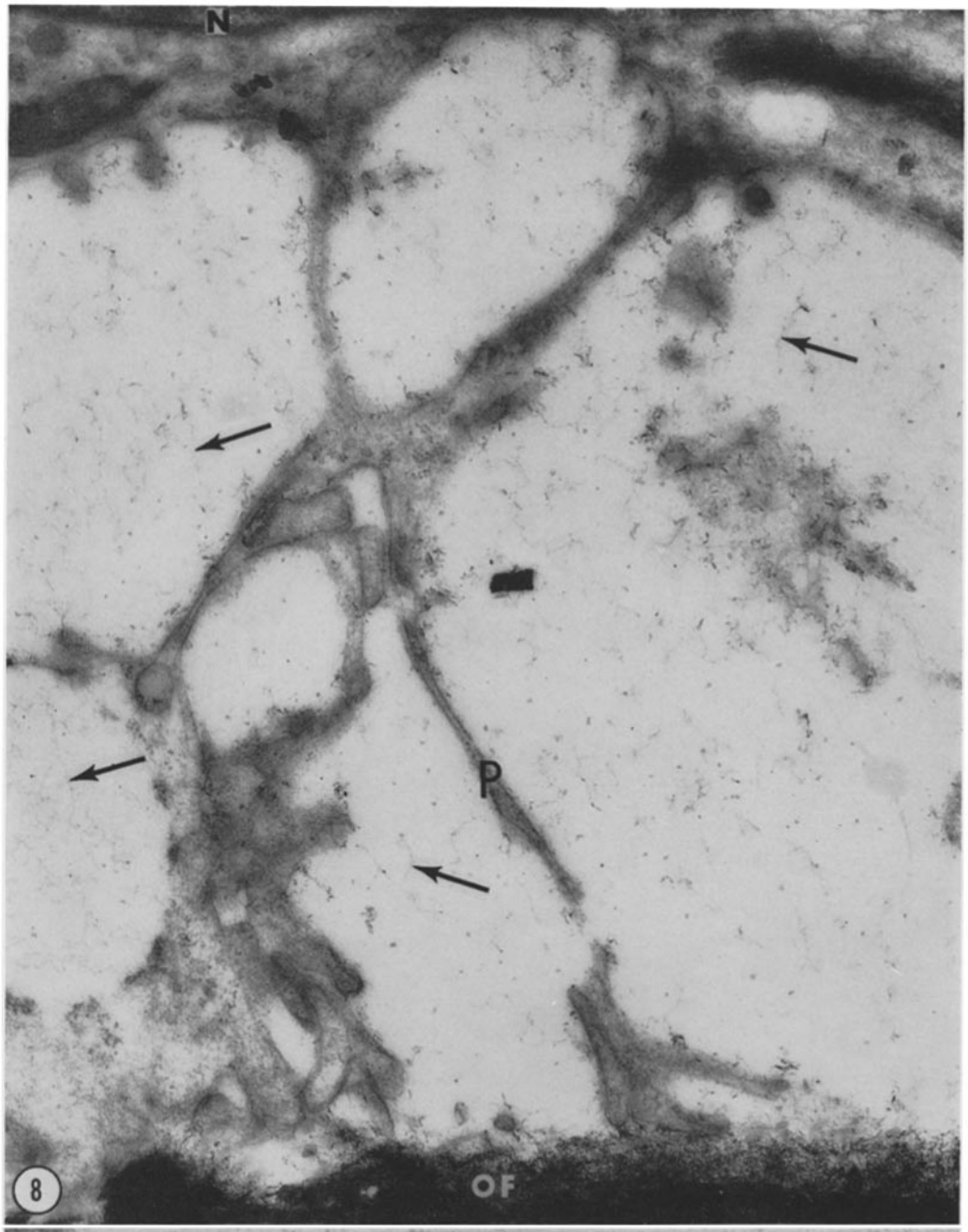
N
P
OF
8
OL
IL
9

FIGURE 10

Electron micrograph of nuclear area of a section of an osteoclast. Parts of four nuclei (*N*) are visible. Golgi membranes and vesicles are seen at *G*. Some mitochondria are pointed out at *M*. The arrows point out characteristic intramitochondrial granules. Bits of rough endoplasmic reticulum can be seen in center of picture. Magnification, 34,000.

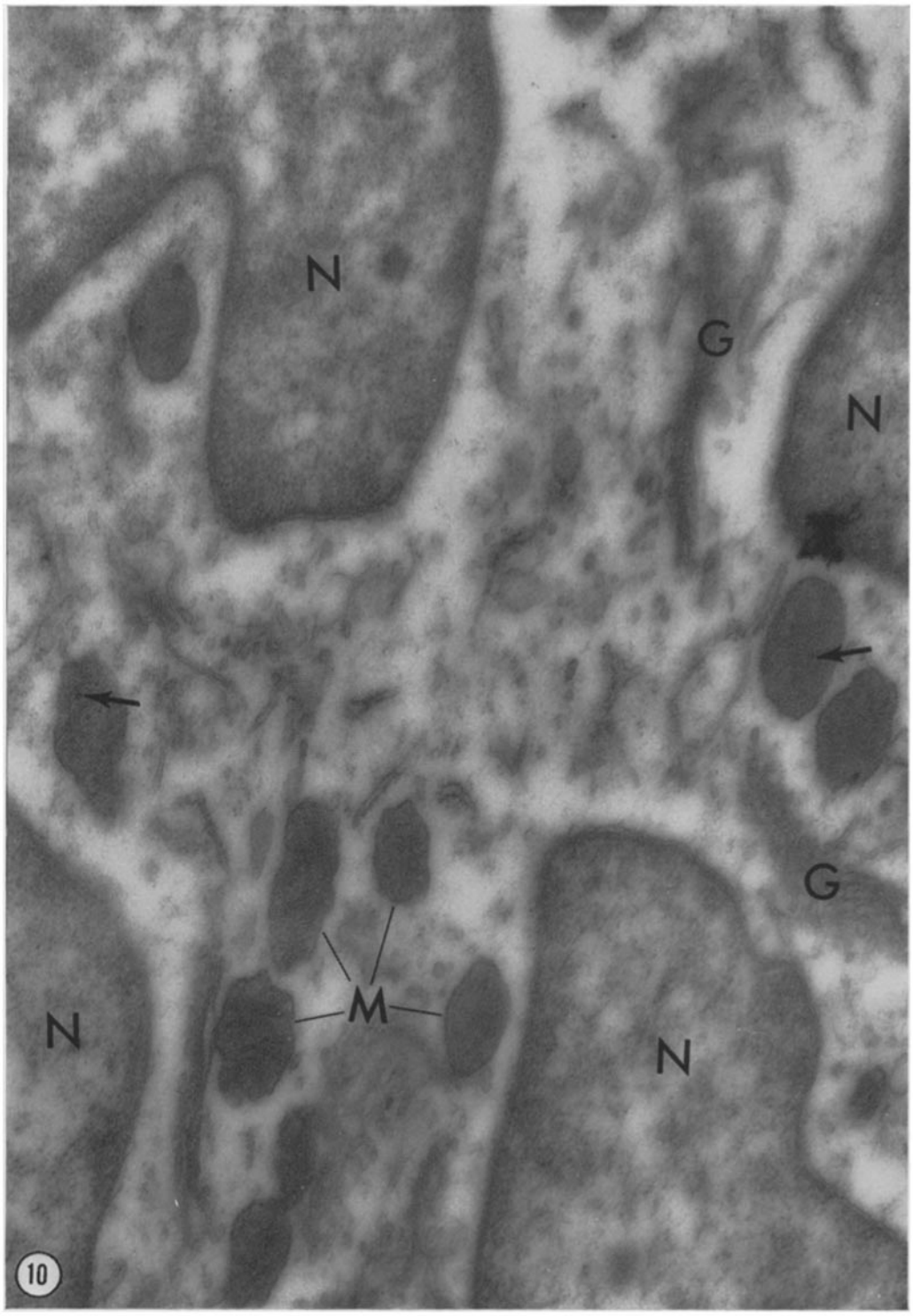
N
G
N
G
M
N
N
10

FIGURE 11

Electron micrograph of a section of an osteoclast showing a particularly high concentration of mitochondria. Note the irregular cristae. Circular profiles can be seen in some of the mitochondria suggesting finger-like projections of the inner mitochondrial membrane. Arrows point out some of the prominent intramitochondria granules. Already at this magnification there is a suggestion of the cluster-like nature of these granules. Magnification, 60,000.

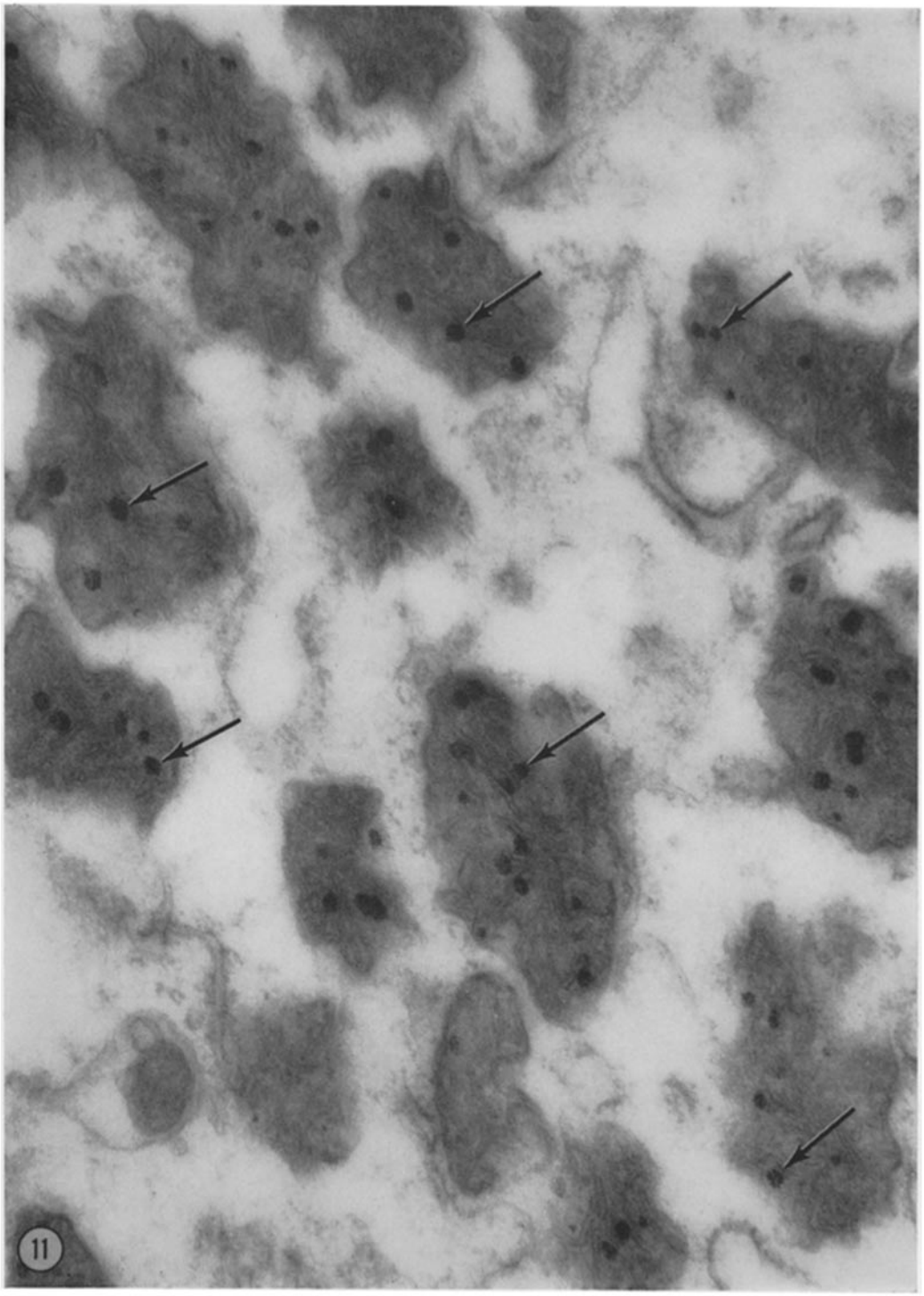
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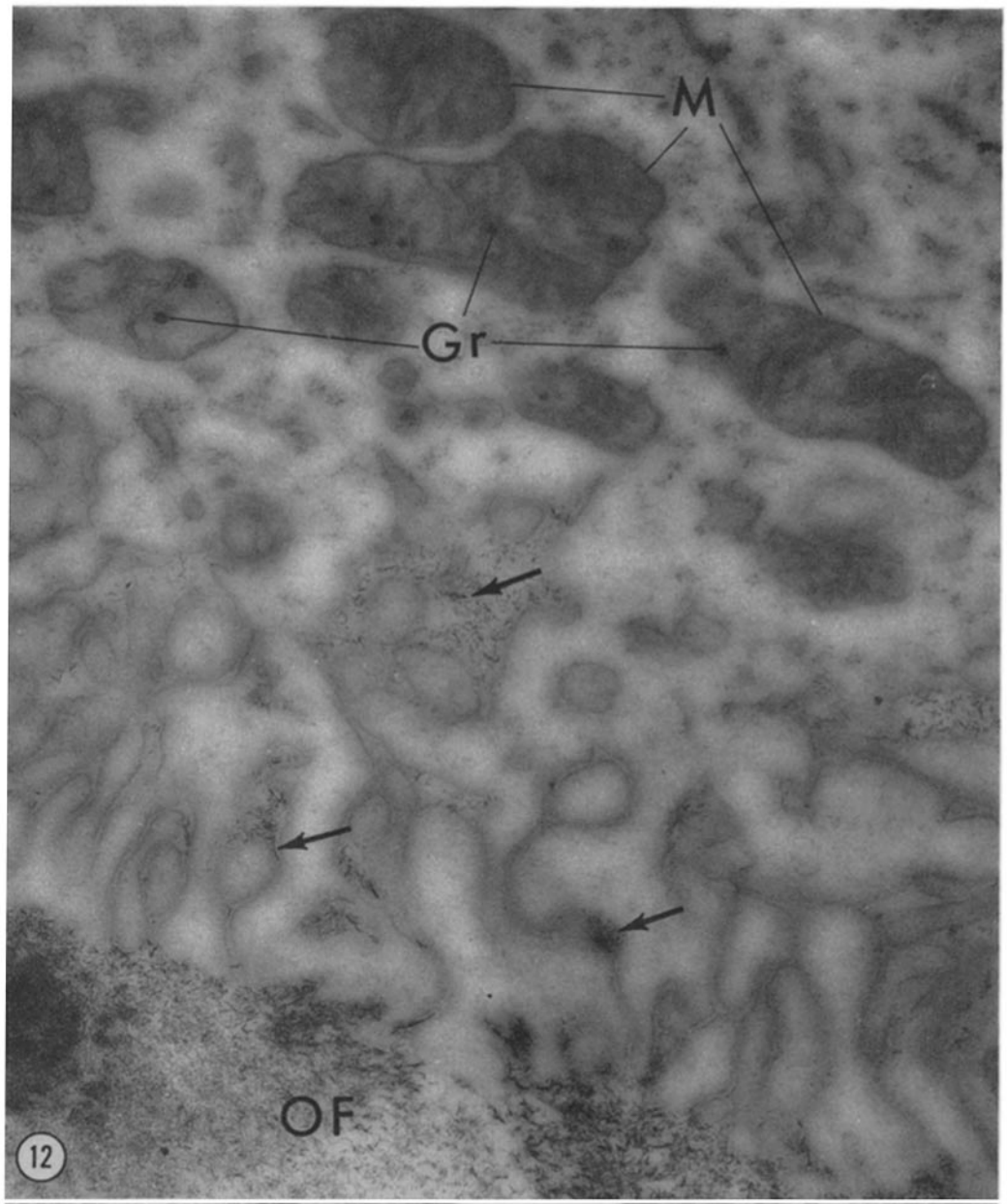
FIGURE 12

Electron micrograph of a portion of an osteoclast. This field shows the osseous fringe (*OF*). The ruffled border containing crystals (arrows) is in intimate contact with the osseous fringe. Mitochondria (*M*) are seen to contain intramitochondrial granules (*Gr*). Magnification, 34,000.

FIGURE 13

High magnification electron micrograph of portions of two mitochondria (*M*). Arrows point to intramitochondrial granules which can be seen to be clusters of still finer granules. Magnification, 150,000.


M
Gr
OF
12
M
M
13

FIGURES 14 and 15

Tracings from electron micrographs of osteoclasts. The solid black areas represent cytoplasm, the stippled areas bone matrix.

FIGURE 14

An example of a tight infolding of the cell membrane. Many vacuoles can be seen toward the interior of the cell. Most of these vacuoles contained crystals.

FIGURE 15

An example of a loose or open infolding of the cell membrane. Many sections of fine cytoplasmic process are visible. Here again, many vacuoles are present deeper in the cell; many undoubtedly communicate with the infolded border.